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Wnt signaling arrests effector T cell differentiation and generates CD8$^+$ memory stem cells

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Self-renewing cell populations such as hematopoietic stem cells and memory B and T lymphocytes might be regulated by shared signaling pathways[1]. The Wnt–β-catenin pathway is an evolutionarily conserved pathway that promotes hematopoietic stem cell self-renewal and multipotency by limiting stem cell proliferation and differentiation[2,3], but its role in the generation and maintenance of memory T cells is unknown. We found that induction of Wnt–β-catenin signaling by inhibitors of glycogen sythase kinase-3β or the Wnt protein family member Wnt3a arrested CD8$^+$ T cell development into effector cells. By blocking T cell differentiation, Wnt signaling promoted the generation of CD44^{low}CD62L^{high}Sca-1^{high}CD122^{high}Bcl-2^{high} self-renewing multipotent CD8$^+$ memory stem cells with proliferative and antitumor capacities exceeding those of central and effector memory T cell subsets. These findings reveal a key role for Wnt signaling in the maintenance of 'stemness' in mature memory CD8$^+$ T cells and have major implications for the design of new vaccination strategies and adoptive immunotherapies.

T cell factor-1 (Tcf-1) and lymphoid enhancer-binding factor-1 (Lef-1) are downstream transcription factors of the Wnt–β-catenin signaling pathway. Tcf-1 and Lef-1 are required for normal thymic T cell development, but less is known about Wnt function in mature T cells[2,4]. Although experiments using a multimerized *Tcf-Lef* binding site reporter system have revealed that Wnt signaling is active in mature CD8$^+$ T cells, the impact of this pathway on this cell population has yet to be fully elucidated[5]. At least three lines of evidence indicate that Wnt signaling might regulate the maturation of post-Thymic T lymphocytes. CD8$^+$ T cells from *Tcf7*$^{-/-}$ mice, which are missing the gene that encodes Tcf-1, show a more differentiated phenotype (CD44^{high} and CD62L^{low}) than wild-type (WT) T cells[6]. In addition, expression of *Lef1* and *Tcf7* decreases with progressive differentiation of CD8$^+$ T cells from naive T cells (T_N cells) to central memory T cells (T_{CM} cells) and effector memory T cells (T_{EM} cells) in humans[7] and mice (**Supplementary Fig. 1**). Finally, high levels of *Ctnnb1* (which encodes β-catenin), *Lef1* and *Tcf7* have been detected in T cells with increased potential to form memory cells *in vivo*[8,9]. Thus, triggering the activities of the Wnt signaling transcription factors Tcf-1 and Lef-1 might promote stem-like self-renewal capacity in mature T cells.

To test the impact of Wnt–β-catenin signaling on mature CD8$^+$ T cells, we primed T_N cells in the presence of the 4,6-disubstituted pyrrolopyrimidine TWS119, a potent inhibitor of the serine-threonine kinase glycogen synthase kinase-3β (Gsk-3β)[10]. Gsk-3β blockade mimics Wnt signaling by promoting the accumulation of β-catenin, the molecule that tethers the Tcf and Lef transcription factors to targeted DNA[2]. TWS119 triggered a rapid accumulation of β-catenin (mean 6.8 ± s.d. 1.7-fold increase by densitometry; $P < 0.05$) (**Fig. 1a**), augmented nuclear protein interaction with oligonucleotide containing the DNA sequences to which Tcf and Lef bind (**Fig. 1b**) and sharply upregulated the expression of *Tcf7* (ref. 11), *Lef1* (ref. 12) and other Wnt target genes including *Jun*[13], *Fzd7* (encoding Frizzled-7)[14], *Nlk* (encoding Nemo-like kinase)[15] (**Fig. 1c**). By contrast, T cell activation in the absence of the Gsk-3β inhibitor resulted in the downregulation of the Wnt–β-catenin signaling cascade (**Fig. 1a–c**). Thus, TWS119 activated the Wnt–β-catenin pathway in naive T cells and reversed the physiological downregulation of *Tcf7* and *Lef1* induced by T cell activation[7].

We sought to assess the effect of Wnt signaling on CD8$^+$ T cell differentiation and proliferation. We stimulated carboxyfluorescein succinimidyl ester (CFSE)-labeled CD8$^+$ T cells from the gp100-specific T cell receptor (TCR)-transgenic mouse, pmel-1 (ref. 16), with its cognate antigen in the presence of titrated doses of TWS119 and analyzed them for the expression of the differentiation markers CD44 and CD62 ligand (CD62L). CD44 expression is known to increase with T cell differentiation, whereas CD62L expression is progressively lost[17]. Treatment of TWS119 increased the frequency of T cells that retained CD62L expression in a dose-dependent manner, indicating that it inhibits CD8$^+$ T cell differentiation (**Fig. 2a**). Notably, 46% of CD8$^+$ T cells cultured in the presence of the highest concentration of Gsk-3β inhibitor failed to upregulate CD44, maintaining a naive CD44^{low}CD62L^{high} phenotype (**Fig. 2a**). Low doses of TWS119 (≤ 1 μM) preserved CD62L expression without affecting T cell proliferation, whereas higher TWS119 concentrations inhibited cell cycling in a dose-dependent manner (**Fig. 2b**). Arrested

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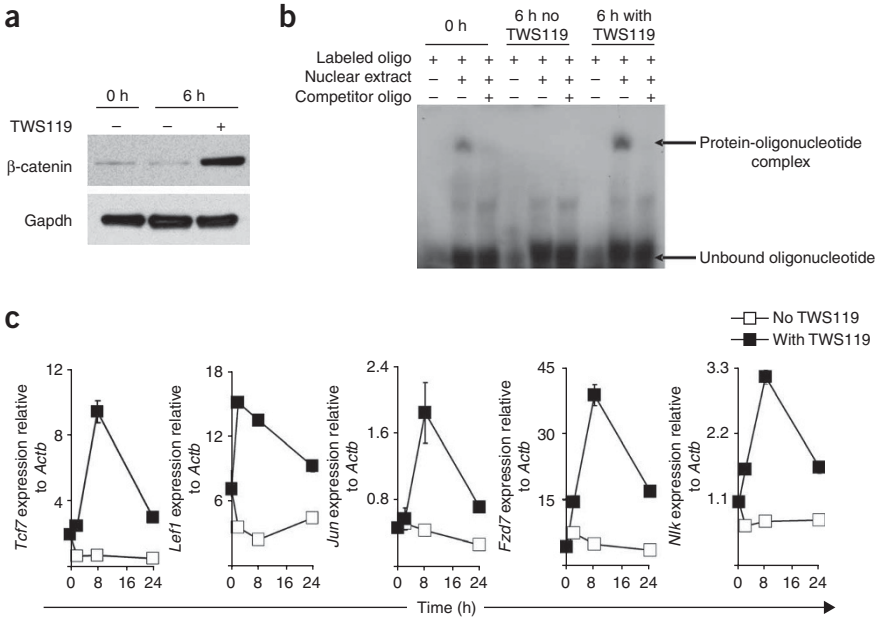

Figure 1 TWS119 activates Wnt signaling in CD8$^+$ T cells. Naive CD8$^+$ T cells were primed *in vitro* with antibody to CD3 (2 μg ml^{-1}) and antibody to CD28 (1 μg ml^{-1}) with or without 7 μM TWS119. (**a**) Western blot analysis of β-catenin and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) in CD8$^+$ T cells treated with or without TWS119. (**b**) Electrophoretic mobility shift assay of nuclear extract from CD8$^+$ T cells treated with or without TWS119 using P^{32}-labeled oligonucleotide probes (oligo) designed from the Tcf and Lef binding region of the Tcf-1 target gene *Fzd7*. Unlabeled oligonucleotide probes were used as competitors. (**c**) Quantitative RT-PCR analysis of the expression of *Tcf7*, *Lef1*, *Jun*, *Fzd7* and *Nlk* in CD8$^+$ T cells treated with or without TWS119. Data are represented as means ± s.e.m. All data are representative of at least two independently performed experiments.

differentiation and proliferation of CD8$^+$ T cells mediated by TWS119 were not secondary to its impact on dendritic cells (DCs) because we observed similar results when we stimulated purified CD8$^+$ T cells in a DC-free system (**Supplementary Fig. 2a**,**b**). Similarly to TWS119, the structurally unrelated Gsk-3β inhibitor 6-bromo-substituted indirubin, BIO[18,19], inhibited T cell differentiation (**Supplementary Fig. 3a**) and induced *Tcf7* and *Lef1* expression (**Supplementary Fig. 3b**). An analog with a greater Gsk-3β kinase inhibitory specificity, BIO-acetoxime[19] retained the observed activity, whereas the *N*-methylated analog (methyl-BIO)[19], which does not block the kinase activity of Gsk-3β, had no effect (**Supplementary Fig. 3a**,**b**). These results are in contrast with those obtained by other researchers using lithium chloride[20], a less active and less specific Gsk-3β inhibitor[19]. Because Gsk-3β regulates several signaling pathways other than the Wnt pathway, we sought to more directly test whether the impact of the pharmacological blockade of Gsk-3β was dependent on mimicking the downstream signals of the Wnt–β-catenin pathway. We primed CD8$^+$ T cells in the presence of Wnt3A, a Wnt protein that has been shown to promote hematopoietic stem cell self-renewal and pluripotency *via* β-catenin accumulation in the cell nucleus[21]. Similarly to TWS119, Wnt3A itself inhibited T cell differentiation and proliferation (**Supplementary Fig. 4**). Thus, T cell proliferation and differentiation could be restrained through the activation of the Wnt–β-catenin pathway by the naturally occurring ligand Wnt3A and by the pharamcologic inhibition of Gsk-3β downstream. Nevertheless, these data do not rule out the possibility that Gsk-3β inhibitors regulate T cell differentiation by affecting other pathways in addition to the Wnt pathway.

We sought to evaluate whether the phenotypic arrest of differentiation of pmel-1 CD8$^+$ T cells that was induced by TWS119 was associated with a block in the acquisition of effector functions. It has been previously shown that differentiating CD8$^+$ T cells lose the capacity to produce interleukin-2 (IL-2) as they acquire the ability to kill target cells and release large amounts of interferon-γ (IFN-γ)[17,22]. We found that TWS119 induced a dose-dependent decrease in T cell–specific killing and IFN-γ release associated with a preservation of the ability to produce IL-2 (**Fig. 2c**). These functional data confirmed our phenotypic findings that TWS119 was a negative regulator of effector T cell (T_{eff} cell) differentiation. We next assessed whether inhibition of T_{eff} cell development is associated with the suppression of eomesodermin (encoded by *Eomes*), a master regulator of CD8$^+$ T cell effector function[23]. We found that exposure of cells to TWS119 abrogated the induced expression of *Eomes* that occurred within 8 h after T cell priming, indicating that TWS119-mediated suppression of the effector program is an early event (**Fig. 2d**). Altogether, phenotypic, functional and molecular data indicated that induction of Wnt signaling during T cell priming results in inhibition of CD8$^+$ T cell differentiation into T_{eff} cells. Given our *in vitro* findings, we tested whether TWS119 can influence the qualities of adoptively transferred pmel-1 CD8$^+$ T cells in response to fowlpox-based gp100 immunization and prevent the induction of highly differentiated, senescent T cells, a major pitfall of current T cell–based vaccines[24]. We found that there were no differences in frequency or numbers of pmel-1 T cells in spleens 6 d after vaccination, but development of T_{eff} cells was inhibited (**Fig. 2e**). More notably, TWS119 effectively altered CD8$^+$ T cell differentiation after immunization, as evidenced by increased numbers of CD44^{high}CD62L^{high} T_{CM} cells in responding CD8$^+$ T cells after vaccination (**Fig. 2e**). However, we cannot ascertain whether the arrest of CD8$^+$ T cell differentiation that we observed *in vivo* was a direct effect of TWS119 on this cell population or instead indirectly mediated through another cell type.

We observed a cell population that expressed low levels of CD44 and high levels of CD62L on the cell surface when we administered TWS119 *in vitro* and *in vivo* (**Fig. 2a**,**e**) but it was unclear whether these cells remained naive after antigen encounter or had entered into a primordial memory state that retained some phenotypic traits of T_N cells. In a mouse model of graft-versus-host disease, other researchers have described a population of CD44^{low}CD62L^{high} memory CD8$^+$ T cells that express high cell surface levels of stem cell antigen-1 (Sca-1), B-cell lymphoma protein-2 (Bcl-2) and common IL-2 and IL-15 receptor β chain (CD122)[25]. Because these cells showed robust self-renewal and the multipotent capacity to generate T_{CM}, T_{EM} and T_{eff} cells, they were designated 'T memory stem cells' (T_{SCM} cells)[25]. We sought to explore whether the CD44^{low}CD62L^{high} pmel-1 T cells that we generated *in vitro* with TWS119 treatment are T_{SCM} cells. We found that the CD44^{low}CD62L^{high} T cells generated after priming with antigen in the presence of TWS119 had undergone up to four divisions, as indicated by CFSE dilution, and uniformly expressed high cell surface levels of the core phenotypic markers of T_{SCM} cells,

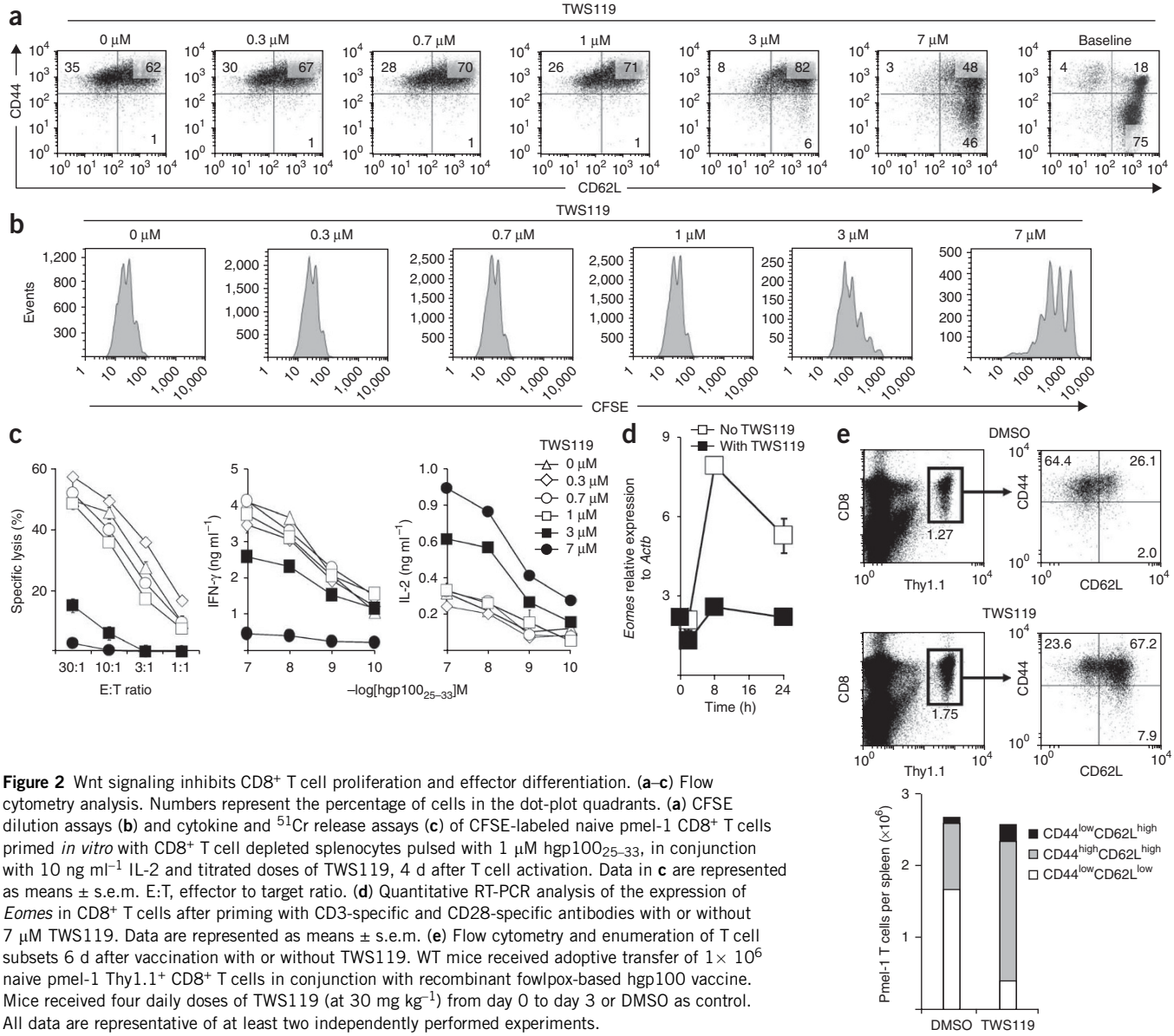

Figure 2 Wnt signaling inhibits CD8+ T cell proliferation and effector differentiation. (**a**–**c**) Flow cytometry analysis. Numbers represent the percentage of cells in the dot-plot quadrants. (**a**) CFSE dilution assays (**b**) and cytokine and ^{51}Cr release assays (**c**) of CFSE-labeled naive pmel-1 CD8+ T cells primed *in vitro* with CD8+ T cell depleted splenocytes pulsed with 1 μM hgp100$_{25–33}$, in conjunction with 10 ng ml^{-1} IL-2 and titrated doses of TWS119, 4 d after T cell activation. Data in **c** are represented as means ± s.e.m. E:T, effector to target ratio. (**d**) Quantitative RT-PCR analysis of the expression of *Eomes* in CD8+ T cells after priming with CD3-specific and CD28-specific antibodies with or without 7 μM TWS119. Data are represented as means ± s.e.m. (**e**) Flow cytometry and enumeration of T cell subsets 6 d after vaccination with or without TWS119. WT mice received adoptive transfer of 1× 10^6 naive pmel-1 Thy1.1+ CD8+ T cells in conjunction with recombinant fowlpox-based hgp100 vaccine. Mice received four daily doses of TWS119 (at 30 mg kg^{-1}) from day 0 to day 3 or DMSO as control. All data are representative of at least two independently performed experiments.

namely Sca-1, CD122 and Bcl-2 (**Fig. 3a**). By contrast, freshly isolated T_N cells expressed these markers in small amounts (**Fig. 3a**). Similarly, Sca-1, CD122 and Bcl-2 were upregulated in CD44^{low}CD62L^{high} pmel-1 T cells generated *in vitro* in the presence of BIO-acetoxime (**Supplementary Fig. 5**) or Wnt3A (**Supplementary Fig. 6**) and *in vivo* after vaccination with gp100 and TWS119 administration (**Supplementary Fig. 7**). These activation and memory T cell markers were not exclusively expressed by T_{SCM} cells but rather defined T_{SCM} cells in the context of cells the were also CD44^{low} and CD62L^{high} (data not shown). Antigen-experienced memory T cells can be distinguished from T_N cells not only by phenotype but also by a number of functional properties, including rapid acquisition of effector functions upon antigen rechallenge[26–28], pronounced cell cycling capacity[29], robust homeostatic proliferation[30] and independence from major histocompatibility complex (MHC) class I for persistence[31] and antitumor activity[32]. We found that, unlike T_N cells, TWS119-induced CD44^{low}CD62L^{high} T cells rapidly released cytokines (IFN-γ and IL-2) upon antigen encounter (**Fig. 3b**), had undergone more cell division after adoptive transfer into a lymphoreplete host (**Fig. 3c**) or into sublethally irradiated or genetically lymphodepleted *Tcra*$^{-/-}$ (T cell–deficient) or *Rag1*$^{-/-}$ (T and B cell–deficient) hosts (**Fig. 3d**), and persisted and mediated tumor destruction ($P < 0.05$) in *B2m*$^{-/-}$ mice (which are MHC class I deficient) (**Fig. 3e**,**f**). Altogether, these findings indicated that Wnt signaling induces the generation of a T_{SCM} cell–like population that possesses the rapid recall ability, proliferative capacity and MHC class I independence that are characteristic of memory but not naive T cells.

The concept of stemness encompasses the capability both to self-renew and to generate more differentiated, specialized cells. We sought to determine the stemness of TWS119-generated CD44^{low}CD62L^{high} T cells by evaluating the fate of these cells after adoptive transfer into sublethally irradiated mice. Four weeks later, we found that high percentages of TWS119-generated cells preserved their original CD44^{low}CD62L^{high} phenotype even after multiple cell divisions, whereas T_N cells rapidly acquired CD44 with progressive CFSE dilution ($P < 0.02$; **Fig. 3g**,**h**). The differentiation of TWS119-induced

CD44^{low}CD62L^{high} T cells was not arrested, however, because they acquired high CD44 expression, albeit at a slower pace than T_N cells (**Fig. 3g,h**). Unlike T_N cells, TWS119-derived CD44^{low}CD62L^{high}Sca-1^{high} T cells robustly proliferated (by 28 d after transfer into sublethally irradiated mice, 95% had undergone cell division) (**Fig. 3h**), enabling their reisolation and transfer to a secondary sublethally irradiated recipient (**Supplementary Fig. 8**). After 4 weeks, we found that the secondarily transferred CD44^{low}CD62L^{high} Sca-1^{high} T cells had again regenerated all T cell subsets. Notably, 43% of the cells retained the T_{SCM} phenotype in spite of a second round of robust proliferation *in vivo* (**Supplementary Fig. 8**). These findings indicated that Wnt signaling promoted the generation of self-renewing, multipotent T_{SCM} cells.

Having established that the CD44^{low}CD62L^{high} T cells generated in the presence of TWS119 are *bona fide* T_{SCM} cells and not merely naive, we sought to compare them with the well-defined memory T cell subsets, T_{CM} cells and T_{EM} cells, in secondary challenge experiments. We adoptively transferred highly purified pmel-1 memory

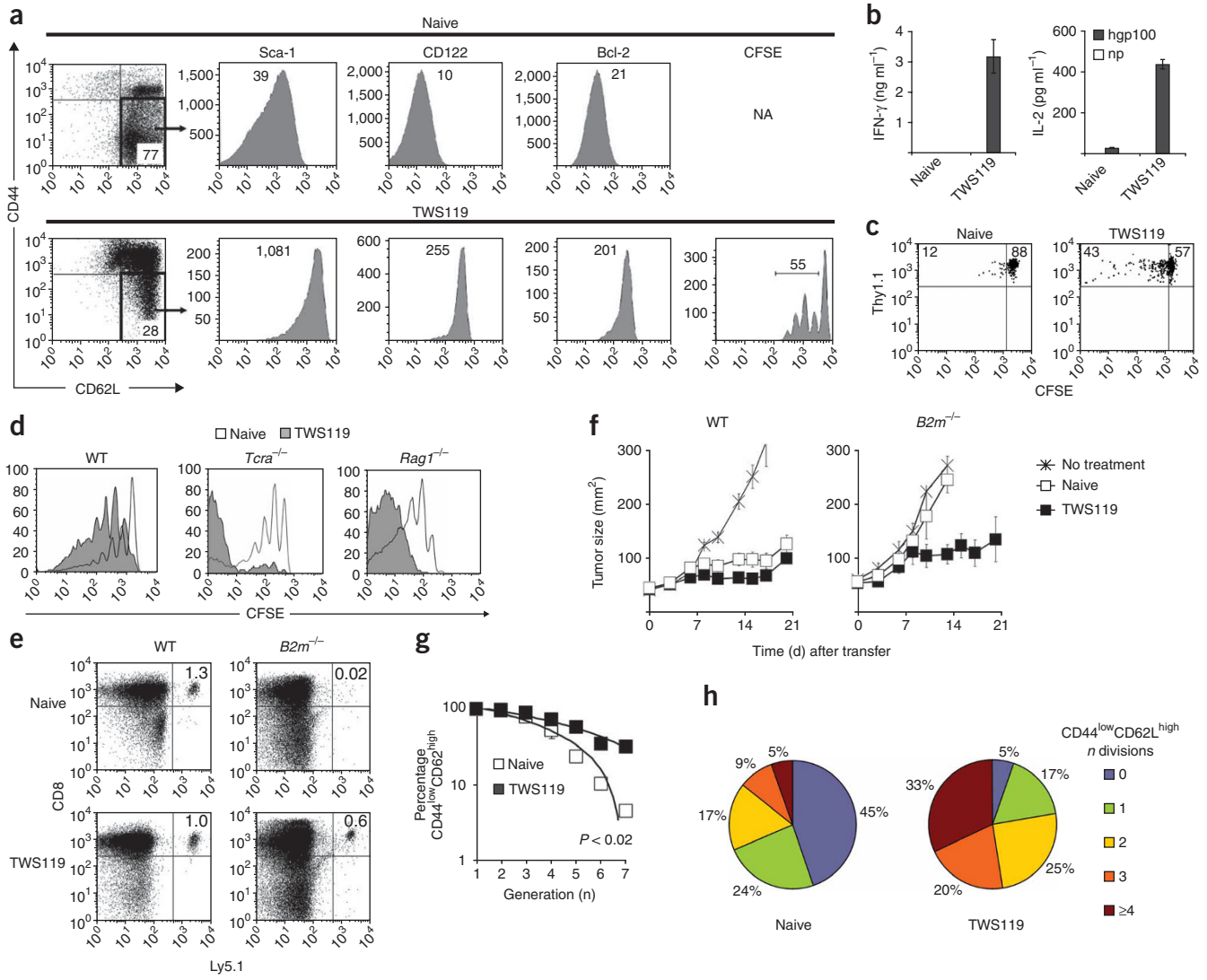

Figure 3 Wnt signaling promotes the generation of T_{SCM} cells. CFSE-labeled, naive pmel-1 CD8$^+$ T cells were primed *in vitro* with CD8$^+$ T cell–depleted splenocytes pulsed with 1 μM hgp100$_{25–33}$, in conjunction with 10 ng ml^{-1} IL-2 and 7 μM TWS119. (**a**) Flow cytometry analysis of TWS119-treated pmel-1 T cells 4 d after T cell activation and freshly isolated naive pmel-1 T cells. Numbers represent the mean fluorescence intensity in the histogram plots of Sca-1, CD122 and Bcl-2 and the percentage of cells in the dot-plot quadrants and the CFSE histogram. NA, not applicable. (**b**) Cytokine release assay of sorted CD44^{low}CD62L^{high} TWS119-treated pmel-1 T cells 5 d after antigenic stimulation and freshly isolated naive pmel-1 T cells. Data are represented as means ± s.e.m. np, nucleoprotein peptide. (**c**,**d**) CFSE dilution of sorted CD44^{low}CD62L^{high} TWS119-treated or freshly isolated naive pmel-1 T cells congenically marked with Thy 1.1$^+$ (**c**) or Ly5.1$^+$ (**d**) pmel-1 T cells 1 month after transfer into WT (**c**) or sublethally irradiated WT, *Tcra*$^{-/-}$,or *Rag1*$^{-/-}$ mice (**d**). Data are shown on Thy 1.1$^+$ (**c**) or Ly5.1$^+$ (**d**) CD8$^+$ lymphocytes. Numbers represent the percentage of cells in the dot-plot quadrants. (**e**) Flow cytometry analysis of sorted TWS119-treated and naive Ly5.1$^+$ pmel-1 T cells 1 month after transfer into WT or *B2m*$^{-/-}$ mice. Numbers represent the percentage of cells in the dot-plot quadrants. (**f**) Tumor size after treatment of myeloablated WT or *B2m*$^{-/-}$ mice bearing B16 tumors established for 7 d. Mice received syngeneic age-matched, lineage-depleted bone marrow cells. On the next day, 1 × 10^6 CD44^{low}CD62L^{high} TWS119-treated or freshly isolated naive pmel-1 T cells were transferred in conjunction with intraperitoneal injection of IL-2. (**g**,**h**) Flow cytometry analysis of CFSE-labeled, sorted CD44^{low}CD62L^{high} TWS119-treated or freshly isolated naive pmel-1 T cells congenically marked with Ly5.1$^+$ 1 month after transfer into sublethally irradiated WT mice. Data are represented as the percentage of CD44^{low}CD62L^{high} cells as a function of CFSE dilution of two independent experiments (**g**) or as fraction of cells with any given number of divisions (**h**). All data are representative of at least two independently performed experiments.

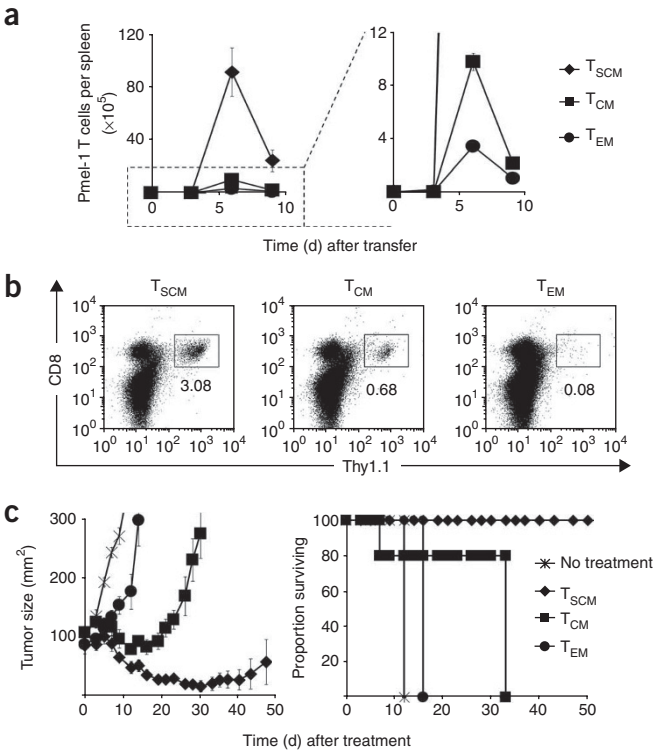

Figure 4 T_{SCM} cells possess enhanced *in vivo* recall response and antitumor activity compared to T_{CM} cells and T_{EM} cells. Pmel-1 naive $CD8^+$ T cells congenically marked with Thy1.1 were primed *in vitro* with splenocytes pulsed with 1 μM $hgp100_{25–33}$, in conjunction with 10 ng ml^{-1} IL-2 with or without 7 μM TWS119. Five days after antigenic stimulation, T_{SCM}, T_{CM} or T_{EM} cells were sorted on the basis of phenotype (T_{SCM}, $CD44^{low}CD62L^{high}$ cells; T_{CM}, $CD44^{high}CD62L^{high}$ cells; T_{EM}, $CD44^{high}CD62L^{low}$ cells). Sublethally irradiated WT mice received 5×10^4 pmel-1 T_{SCM}, T_{CM} or T_{EM} cells in conjunction with a recombinant vaccinia virus encoding hgp100 and intraperitoneal injection of IL-2. (**a**) Absolute numbers of adoptively transferred pmel-1 T cells (identified by $CD8^+Thy1.1^+$ lymphocytes) in the spleens of treated mice. Data are represented as means ± s.e.m. (**b**) Flow cytometry analysis for the expression of CD8 and Thy1.1 one month after adoptive transfer of T cell memory subsets. (**c**) Tumor size and survival of sublethally irradiated WT mice bearing B16 tumors established for 10 d ($n = 5$ for all groups) receiving 4×10^4 pmel-1 T_{SCM}, T_{CM} or T_{EM} cells in conjunction with a recombinant vaccinia virus encoding hgp100 and exogenous IL-2. Data are represented as means ± s.e.m. All data shown are representative of at least two independently performed experiments.

T cell subsets into sublethally irradiated mice in combination with recombinant vaccinia-based gp100 vaccine and IL-2 treatment. As previously reported by our group and others[33,34], the T_{CM} cells proliferated more than T_{EM} cells (**Fig. 4a**). T_{SCM} cells, however, robustly expanded by approximately 200-fold in the spleen alone (**Fig. 4a**). These levels of expansion were approximately ten times higher than those of T_{CM} cells and approximately 30 times higher than those of T_{EM} cells (**Fig. 4a**). Furthermore, T_{SCM} cells showed an enhanced survival capacity, as revealed by the frequencies of pmel-1 T cells in the spleens of vaccinated mice 1 month after transfer (**Fig. 4b**). T cell proliferative and survival capacities have been correlated with tumor responses in mice and humans receiving adoptive T cell–based therapies[35,36]. To assess whether the enhanced replicative capability of T_{SCM} cells would result in superior antitumor activity, we adoptively transferred limiting numbers of pmel-1 memory T cell subsets into sublethally irradiated hosts bearing B16 tumors, in combination with gp100 vaccine and IL-2. As we previously reported[33], T_{CM} cells conferred superior antitumor immunity compared with T_{EM} cells (for tumor regression, $P < 0.01$; for overall survival, $P = 0.0644$) (**Fig. 4c**). Notably, minuscule numbers (4×10^4) of T_{SCM} cells were able to trigger the destruction of bulky tumors (1 cm^3 containing $\sim 1 \times 10^9$ cells) and improve survival (T_{SCM} cells versus T_{CM} cells, $P < 0.005$; T_{SCM} cells versus T_{EM} cells, $P < 0.005$; **Fig. 4c**). Thus, adoptive transfer of T_{SCM} cells in combination with tumor antigen vaccination and exogenous IL-2 produced a far more robust and therapeutically noteworthy secondary response compared with the other memory T cell subsets.

Arrest of lymphocyte differentiation to maintain long-lived, self-renewing antigen-experienced T cells with stem cell-like properties has been postulated as the basis of the continual generation of effector T cells[37], but the transcription factors that regulate this process have not been fully elucidated. Although the physiologic role of Wnt signaling in post-Thymic T cell development remains unknown, our data indicate that Wnt can regulate the stemness of $CD8^+$ T cells by suppressing their differentiation into T_{eff} cells. Other circumstantial evidence supports our findings. We recently reported that high levels of the Wnt transcription factors *Tcf7* and *Lef1* were expressed by T cells whose antigen-induced development into T_{eff} cells had been arrested using IL-21 in the culture medium[8]. Conversely, *Tcf7*$^{-/-}$ mice reportedly have an increased frequency of $CD44^{high}CD62L^{low}$ T_{EM} cells[6]. Paralleling our own observations in $CD8^+$ T cells, in $CD4^+$ T cells expression of a stabilized form of β-catenin in $CD4^+$ T cells inhibited the proliferation and effector function of the cells in a model of inflammatory bowel disease[38]. Furthermore, $CD4^+$ T cell memory precursors overexpressed *Ctnnb1* and *Tcf7* as well as *Bcl2* and *Il2rb* (ref. 9). These factors were all implicated in the formation and maintenance of T_{SCM} cells described here.

Our findings also have parallels in stem cell biology, where Wnt signaling has a pivotal role in promoting self-renewal while limiting proliferation and differentiation[2,3]. Hematopoietic stem cells exposed to an environment in which Wnt signaling was inhibited by overexpression of Dickkopf1 in the stem cell niche were more proliferative than those in WT mice, but they had lost their capacity for self renewal, as reflected by their inability to reconstitute hosts after serial transplants[3]. T cell lineage commitment to effector versus memory subsets has recently been linked to the asymmetrical segregation and inheritance of proteins that control cell fate specification[39]. Although the role of Wnt in the asymmetric division of $CD8^+$ T cells is unknown, unequal localization and activities of Wnt signaling components, including β-catenin, have been implicated in cell fate specification in *Caenorhabditis elegans*[40].

The ability to pharmacologically induce T_{SCM} cells has considerable implications for the field of adoptive immunotherapy. The findings that small numbers of T_{SCM} cells in conjunction with specific vaccination and IL-2 therapy can cause the regression of large established vascularized tumors might reduce the cost and complexity of highly effective therapies based on the adoptive transfer of antitumor T cells[35]. Using existing technology[36], it may be possible to genetically engineer the human counterpart of $CD8^+$ T_{SCM} cells to express T cell receptors or chimeric antigen receptors while pharamacologically mimicking Wnt signaling. This might ultimately allow for the widespread application of adoptive immunotherapy based on multipotent, highly proliferative, tumor-specific T_{SCM} cells. Finally, the modulation of Wnt signaling to induce long-term T cell memory might be crucial for T cell–based vaccines designed to target intracellular pathogens. Data indicate that protection against intracellular pathogens correlates with the induction and maintenance of T_{CM} cell

responses, but these responses have not been reproducibly obtained with current vaccination strategies[24,28,34]. *In vivo* administration of small molecule agonists of Wnt signaling might be useful to consistently achieve this goal.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

L.G. designed, performed and analyzed experiments and wrote the paper; X.-S.Z. D.C.P., Y.J., C.S.H., Z.Y., C.W., A.B., L.C., L.M.G., C.M.P. and P.M. performed experiments; and N.P.R. designed experiments and wrote the paper.

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ONLINE METHODS

Mice and tumor lines. Pmel-1 TCR-transgenic mice (Jackson Laboratories) and pmel-1 ly5.1 double-transgenic mice and pmel-1 Thy1.1 double-transgenic mice were previously described[16,17]. We obtained B16 (H-2D^b), a gp100$^+$ murine melanoma, and MCA-205 (H-2D^b), a gp100$^-$ sarcoma, from the National Cancer Institute Tumor Repository.

Antibodies, flow cytometry and cell sorting. We purchased all antibodies from BD Biosciences. We performed flow cytometry acquisition on a BD FACSCanto or BD FACSCanto II flow cytometer. We analyzed samples with FlowJo software (Tree Star). We sorted naive and T cell memory subsets with the BD FACSAria cell sorter.

Pharmacologic inhibitors of Gsk-3β and Wnt3A. We reconstituted TWS119, BIO, BIO-acetoxime, Methyl-BIO (EMD Biosciences) and Wnt3a (R&D Systems) according to the manufacturer's instructions and used them in experiments *in vitro* or *in vivo* at the various doses.

***In vitro* activation of CD8$^+$ T cells.** We stimulated Pmel-1 splenocytes with 1 μM hgp100$_{25–33}$ peptide (Anaspec) in culture medium containing 10 ng ml^{-1} IL-2 (Chiron Corporation). We activated enriched CD8$^+$ T cells isolated from the splenocytes of wild-type mice on plates coated with 0.05–2 μg ml^{-1} CD3-specific antibody and 1 μg ml^{-1} of soluble CD28-specific antibody in culture medium containing 10 ng ml^{-1} IL-2. We separated CD8$^+$ T cells from non-CD8$^+$ T cells with a MACS negative selection kit (Miltenyi Biotech).

Cytokine release and cytolytic assays. We pulsed 2.5×10^4 MCA-205 cells with 1 μM hgp100$_{25–33}$ or irrelevant influenza nucleoprotein peptide and incubated them overnight with T cells at a 1:1 ratio at 37 °C. We analyzed the supernatants by mouse IFN-γ and mouse IL-2 ELISA kits (Pierce-Endogen). For ^{51}Cr release assays, we pulsed MCA-205 target cells with 1 μM hgp100$_{25–33}$ or nucleoprotein peptide and 100 μCi of ^{51}Cr (Amersham Biosciences) for 2 h at 37 °C. We incubated labeled cells (1×10^3 cells) with T cells at ratios indicated in **Figure 2c** for 4 h at 37 °C. We determined maximal and spontaneous ^{51}Cr releases by incubating 1×10^3 labeled targets in either 2% SDS or medium.

Adoptive cell transfer. We performed adoptive cell transfer, immunization with recombinant poxvirus-based vaccines and tumor experiments as previously described[16,17,32]. We used C57BL/6, *Tcra*$^{-/-}$, *Rag1*$^{-/-}$ (Jackson Laboratories) and *B2m*$^{-/-}$ (Taconic) mice as recipients. We conducted all mouse experiments with the approval of the National Cancer Institute Animal Use and Care Committee.

Enumeration of adoptively transferred cells. We killed mice on day 6 (**Fig. 2e**) or days 3, 6 and 9 (**Fig. 4a**). We collected spleens and homogenized them into a single-cell suspension using the rubber end of a 3-cc syringe and a 40-μm filter cup. We enumerated samples by trypan blue exclusion and analyzed the cells by flow cytometry for CD8 and Thy.1 expression. We calculated the absolute number of pmel-1 cells by multiplying the splenocyte count by the percentage of CD8$^+$Thy1.1$^+$cells.

Carboxyfluorescein succinimidyl ester proliferation assays. We used CD8$^+$ T cells labeled with 1 μM CFSE (Molecular Probes) in adoptive experiments or stimulated them *in vitro* as described above.

Real-time reverse transcription-PCR. We isolated RNA with the RNeasy mini kit (Qiagen). We generated complementary DNA by reverse transcription (Applied Biosystems). We performed real-time PCR for all genes with commercially available probes and primers (Applied Biosystems) and a Prism 7900HT (Applied Biosystems). We calculated the levels of gene expression relative to the housekeeping gene encoding β-actin (*Actb*).

Detection of β-catenin by western blot analysis. We lysed cells in RIPA buffer (Cell Signaling Technology) with protease inhibitor. We quantified the protein concentration by Bio-Rad protein assay. We separated 20 μg of total protein on a 4–12% SDS-PAGE gel followed by standard immunoblotting with antibody to β-catenin (BD Bioscience), antibody to Gapdh (Chemicon International) and horseradish peroxidase–conjugated goat antibodies to mouse IgG (Santa Cruz Biotechnology).

Electrophoretic mobility shift assay. We isolated nuclear extracts with the nuclear extract kit (Active Motif) and then incubated them (5 μg protein) with polydeoxyinosinic-deoxycytidylic acid (1 μg μl^{-1} in 10 mM Tris-HCL, 1 mM EDTA, pH 8.0), 5× Binding Buffer (Promega) and γ^{32}P-ATP–labeled oligonucleotide probes (sense: 5′-CACAGAGAAAACAAAGCGCGCTATT-3′; antisense: 5′-AATAGCGCGCTTTGTTTTCTCTGTG-3′), containing the Tcf and Lef binding motif 5′-AACAAAG-3′. We used unlabeled oligonucleotide probes as competitors. We incubated samples for 30 min at 20 °C and loaded them onto the gel (NuPAGE 4–12% or Bis-Tris gels, Invitrogen). We blotted the gel onto a nitrocellulose membrane and exposed the membrane to X-ray film.

Statistical analyses. We compared tumor slopes with the Wilcoxon rank-sum test. We used the Kaplan-Meier method to analyze survival.